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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of Kathleen C.M. Campbell
Serial No. 09/911,195
Filed July 23, 2001
Confirmation No. 2942
For THERAPEUTIC USE OF D-METHIONINE TO REDUCE THE TOXICITY OF NOISE
Examiner Rebecca Cook

Art Unit 1614

DECLARATION OF KATHLEEN C. M. CAMPBELL UNDER 37 CFR 1.132

TO THE COMMISSIONER OF PATENTS AND TRADEMARKS,

SIR:

I, Kathleen C.M. Campbell, hereby declare and state as follows:

1. I reside at 11941 Clearspring Drive, Glenarm, Illinois 62536.

2. I received a Doctor of Philosophy in Audiology/Hearing Science from the University of Iowa in 1989.

3. I am currently a Professor and the Director of Audiology Research in the Division of Otolaryngology, Department of Surgery at the Southern Illinois University School of Medicine in Springfield, Illinois.

4. I am the named inventor of the subject application, which claims methods for treating or preventing ototoxicity in a patient exposed to noise for a time and at an intensity sufficient to result in ototoxicity.

5. I am first author of Campbell et al., "D-Methionine provides excellent protection from cisplatin ototoxicity in the rat," Hearing Research, 102, 90-98 (1996), which the Office has cited against the claims of the subject application.

6. I have reviewed the Office actions dated January 22, 2003; July 15, 2003; September 29, 2003; and August 23, 2004 in the subject application and studied the disclosure of the Campbell et al. reference and the Kopke provisional application (U.S. Serial No. 60/069,761).

7. I am providing this Declaration to address whether the Campbell et al. reference would have motivated one skilled in the art at the time the claimed invention was made to employ methionine for the prevention or treatment of ototoxicity in a patient exposed to noise for a time and at an intensity sufficient to result in ototoxicity. After reviewing the Campbell et al. reference and the comments made in the Office actions referenced above, it is my opinion that the Campbell et al. reference does not provide the necessary motivation to lead one of ordinary skill in the art to the subject matter of the invention defined by claims 1, 20-29 and 32-41.

8. The Campbell et al. reference describes the administration of D-methionine to male rats for the prevention of ototoxicity caused by cisplatin, a chemotherapeutic agent having known ototoxic effects. The reference is entirely devoid of any mention or implication that D-methionine could or would protect against noise-induced hearing loss. Significantly, the reference discloses that D-methionine protected not only against cisplatin-induced hearing loss but also against cisplatin-induced nephrotoxicity and weight loss, neither of which are factors in noise-induced ototoxicity, and which therefore imply different mechanisms of toxicity and protection. Moreover, the male Wistar rats that were the experimental subjects of the reference were kept in an environment wherein the noise level did not rise to an ototoxic level.

9. The Campbell reference includes some analysis as to the possible mechanisms by which cisplatin ("CDDP") causes ototoxicity, nephrotoxicity and weight loss, and the manner in which D-methionine may counter the effect of CDDP. Nothing in the reference suggests that noise causes hearing loss by a mechanism in any

way similar to the effect of CDDP. In fact, the reference says nothing about noise-induced ototoxicity.

10. Having conducted substantial research in the field of ototoxicity, I am familiar with the literature relating to ototoxicity as resulting from various causes, including CDDP and noise. There are substantial differences in the mechanisms by which hearing loss is induced by noise and by which hearing loss is induced by cisplatin. The differences in mechanism between hearing loss induced by noise and ototoxicity caused by administration of CDDP are evidenced by changes in cells of the ear upon exposure to these agents. These differences are evident in both the stria vascularis and the organ of Corti.

11. Ototoxic noise exposure causes extracellular strial edema with gaps between the marginal cells at the lumen (Duvall et al 1974, Lipscomb et al 1977). Noise damage does not cause mitochondrial migration in the marginal cells but can decrease the number of mitochondria (Johnsson and Hawkins (1972)). In addition, noise exposure can cause pyknotic marginal cell nuclei with increased amounts of chromatin (Johnsson and Hawkins 1972 Duvall et al 1974). Possibly the most remarkable difference from ototoxicity due to cisplatin administration is that the most commonly noted changes to the stria vascularis following noise exposure in animals are a variety of vascular changes (Johnsson and Hawkins 1972, Duvall et al 1974, Lipscomb et al 1977, Santi and Duvall 1978, Vertes et al 1979, Prazma et al 1983, Shaddock et al 1984). Noise is known to cause temporary vasoconstriction in the auditory system (Quirk and Seidman 1995) and some hearing loss may be secondary to reperfusion injury. Further, some studies have hypothesized that outer hair cell loss and hearing loss due to noise are, at least in part, a consequence of strial vascular changes (Seidman et al 1993). Notably, noise can induce microlesions in cochlear hair cell plasma membranes allowing calcium influx. (Mulroy M. Hendry W. 1997) Additionally, noise can rupture Reissner's membrane (Duvall et al 1974) and can rupture or cause holes in the reticular lamina (Duvall et al. 1974, Voldrich and Ulehova

1980, Bohne et al. 1984). These types of damage could result in a mixing of perilymphatic and endolymphatic fluids with direct chemical toxicity to surrounding cells. Furthermore, noise can cause glutamate excitotoxicity at the synapse of the spiral gaglion cells, which can cause acute swelling depending on the AMPA/kainite type of receptors together with a disruption of the postsynaptic structutes. The second phase of excitotoxicity that can develop from noise injury is a cascade of metabolic events triggered by the entry of calcium ions, which can cause neuronal death in the spiral ganglion. (Pujol and Puel 1999) Finally, noise exposure induces expression of heat shock proteins in the cochlea (Lim et al. 1996) and changes cochlear gene expression. (Ryan et al. 1996)

12. In contrast, generally, cisplatin does not cause the above cell changes caused by noise injury. Cisplatin can damage cells by causing DNA intra- and interstrand cross links preventing cell replication. (Roberts and Pera 1983) Additionally, cisplatin can damage normal cells by binding to the L-methionine in protein, thus, disrupting the protein and damaging or killing cells. (Lempers and Reedijk 1990) In the auditory system, cisplatin causes different effects than noise injury does. For example, in the stria vascularis, cisplatin causes intracellular edema with bulging and compressed marginal cells along the lumen (Meech et al 1997, Campbell et al 1999). These strial changes include cystic degeneration and protrusions into the endolymphatic duct followed by cell death. (Kohn et al. 1986; Tange and Vuzevski 1984) Further, in strial marginal cells, cisplatin causes migration of mitochondria from the subnuclear to the supranuclear sections of the marginal cells but without an overall loss of numbers of mitochondria (Meech et al 1997, Campbell et al 1999). For marginal cell nuclei, cisplatin can cause degradation of the nuclear envelope and a light appearance of the marginal cell nuclei with apparent loss of chromatin and sometimes missing nucleolus (Meech et al 1997, Campbell et al 1999). In addition, the cuticular plate may soften and more lysosomal bodies may be present in the OHC's apical portion. (Estrem 1981) However, in contrast to noise injury, vascular changes in the stria vascularis have not been reported as a result of cisplatin exposure.

13. In addition, the different ototoxicities exhibit different clinical behaviors. Cisplatin can induce systemic toxicity including neurotoxicity, gastrointestinal toxicity, and peripheral neuropathy in addition to hearing loss, while high-level noise exposure specifically causes hearing loss. Cisplatin can induce delayed hearing loss which arises long after exposure, sometimes after a period of months; and the hearing loss is almost always irreversible. High-level noise is known to cause both temporary threshold shift and permanent threshold shift. However, unlike cisplatin, noise does not induce hearing loss that is first manifested months after the exposure.

14. As discussed in the Campbell et al. reference, the mechanism by which D-methionine is believed to protect against cisplatin-induced ototoxicity teaches away from predicting any otoprotective action against noise-induced ototoxicity. The Campbell et al. reference reports that D-methionine most likely protects against cisplatin-induced ototoxicity by binding to the cisplatin:

> It is logical that free D-Met may preferentially bind to CDDP [i.e., cisplatin] because of the steric hindrance of the protein bound sulfur groups. This protection could occur by preferential binding of the CDDP to D-Met or perhaps D-Met could reverse the Pt binding to the protein bound methionine and glutathione as do other sulfur containing compounds. . . . D-Met binding to CDDP may also protect free L-methionine (L-Met), an essential amino acid. (Campbell et al., p. 95, col. 1.)

15. One skilled in the art would not be led by the Campbell et al. reference to try D-methionine for treating noise-induced ototoxicity because one skilled in the art would not necessarily infer that a treatment effective against cisplatin-induced ototoxicity would also be effective for treating noise-induced ototoxicity. For example, although sodium thiosulfate, fosfomycin, and diethyldithiocarbamate have been shown to protect against cisplatin-induced hearing loss in animals, so far as I am aware none of these agents has ever been reported or apparently even investigated as protecting against noise-induced hearing loss. Undoubtedly if one of these agents had been found to protect against noise-induced hearing

loss, the findings would have been published and would probably have received much attention in the literature, because such findings would have potentially significant clinical impact. If an agent's otoprotection against cisplatin-induced hearing loss motivated one skilled in the art to use that agent to protect against noise-induced hearing loss, it would be logical that all of sodium thiosulfate, fosfomycin, and diethyldithiocarbamate would at least have been tested for that purpose. Based on my familiarity with the field, I would almost certainly be knowledgeable of any testing of these agents for such purpose. Moreover, so far as I am aware, no evidence of testing exists, which supports the conclusion that one skilled in the art would have been motivated by evidence of D-methionine's protection against cisplatin-induced ototoxicity to use D-methionine to achieve otoprotection against noise-induced ototoxicity. Accordingly, due to evidence of different therapeutic mechanisms for different anti-ototoxic agents, a person skilled in the art would not have been led by Campbell et al. to the use of methionine as a protective agent for hearing loss caused by noise.

16. Furthermore, of the two other most promising agents for protection from noise-induced hearing loss, one (acetyl-L-carnitine) to my knowledge has never been reported as protecting against cisplatin-induced hearing loss (and therefore was probably not tested for that use) and the other (N-acetyl cysteine (NAC)) has not been demonstrated to be effective against cisplatin ototoxicity. Only one study has addressed *in vivo* testing, but NAC was only one component of an anti-oxidant mixture comprising alpha-tocopherol acid succinate, ascorbic acid, glutathione and N-acetyl cysteine. Alpha-tocopherol acid succinate, and ascorbic acid, have each independently been shown to be protective against cisplatin-induced ototoxicity. Consequently, it cannot be determined if NAC itself contributed to any otoprotection observed. No studies in the literature have tested NAC alone as an otoprotective agent in an *in vivo* model. *In vitro* studies have produced variable results but the results of *in vitro* studies must be interpreted with caution. Because NAC is a sulfur containing nucleophile, *in vitro* studies could allow NAC to directly interact with the cisplatin, and thus deactivate

the cisplatin prior to cellular uptake, a mechanism that may be irrelevant *in vivo* or if it did occur *in vivo* could cause significant anti-tumor interference prohibiting clinical relevancy. No studies actually testing whether or not NAC inhibits cisplatin anti-tumor activity are in the literature. Additionally, NAC has been shown to exacerbate aminoglycoside-induced ototoxicity, (Bock et al., 1983). These teachings further demonstrate that an agent's protective action against hearing loss caused by one type of stressor cannot necessarily be used to predict whether it will reduce or exacerbate hearing loss secondary to a different cause.

17. U.S. provisional application serial no. 60/069,761 (Kopke et al.) teaches prevention or reversal of hearing loss induced by cisplatin and noise through support of the inner ear's antioxidant defenses by

> increasing antioxidant enzyme levels in the inner ear through the application of agents such as the adenosine agonist R-PIA or other similar agents, or through the application of antiapoptotic agents or trophic factors (growth factors) which may also upregulate antioxidant enzyme levels. (page 7, lines 14-16)

In addition, Kopke et al. teach that an increase in antioxidant levels in the inner ear is "aimed at increasing inner ear glutathione (GSH) levels." (page 7, lines 17-18) Moreover, the provisional application teaches that various sulfur compounds (e.g. L-2-oxothiazolidine-4-carboxylic acid (OTC), L-N-acetylcysteine (L-NAC), methionine and S-adenosyl-L-methionine (SAMe)) can act as substrates for GSH synthesis when in combination with an agent which upregulates the antioxidant enzyme activity such as R-PIA. (page 7, line 20 to page 8, line 6) Thus, Kopke et al. teach an agent which upregulates an antioxidant enzyme in combination with a sulfur compound which is a substrate for GSH synthesis. However, there is no suggestion that methionine as such can be effective against noise-induced hearing loss, or any other form of hearing loss. According to the teachings of the Kopke provisional, it is upregulation of antioxidant enzyme activity which is effective, e.g., as accomplished by administration of R-PIA. Generally,

upregulation of an enzyme is related to an increase of ligand/receptor interactions followed by an increase in the number of available receptors. This increase in receptors stimulates the synthesis of the enzyme by increasing the concentration of mRNA for the particular enzyme and thereby increasing the concentration of the enzyme. By increasing the concentration of the enzyme, the product of the enzymatic reaction is also increased as long as there is a sufficient substrate concentration. A sulfur compound is provided on a supplemental basis merely as a substrate for glutathione synthesis. Thus, sulfur compounds by themselve do not upregulate the antioxidant enzymes.

18. Although as stated above, U.S. provisional application serial no. 60/069,761 (Kopke et al.) teaches that L-NAC is a substrate for GSH synthesis, taken in context, Kopke et al. teach the combination of a substrate for GSH synthesis and an agent which upregulates the antioxidant enzyme activity. In addition, Kopke et al. do not specify whether the combination is specific for hearing loss induced by a chemotherapeutic agent, aminoglycoside antibiotic, noise or closed head injury. As described above, the physiological effects of cisplatin administration and noise injury are very different. Accordingly, a person of ordinary skill would have discounted the teachings of Kopke et al. with respect to L-NAC due to their knowledge of the art that showed NAC has not been demonstrated to be effective against cisplatin-induced ototoxicity and exacerbated aminoglycoside-induced ototoxicity.

19. Furthermore, the effectiveness of similar compounds for a given insult or a compound for a variety of insults can differ greatly. For example, as described above L-NAC is effective for treatment of noise-induced hearing loss, but it is unknown whether it is effective for treatment of cisplatin-induced hearing loss and it exacerbates aminoglycoside-induced hearing loss. Moreover, methionine and related compounds (e.g, D-methionine, L-methionine, methyl-L-methioninate, ethyl-L-methioninate, N-acetyl-D,L-methionine) are the most effective agents against nephrotoxicity associated with cisplatin administration as compared to a

number of other agents tested, many of which are sulfur-containing nucleophiles. (Jones et al. 1989) In addition, upon testing in my laboratory of many sulfur containing compounds against cisplatin-induced ototoxicity by administering 75 mg/kg of the protective agent 30 minutes prior to administration of 16 mg/kg of CDDP in male Wistar rats, either the agents did not exhibit the pronounced ototoxic protective effect of D-methionine or they were too toxic to continue testing. The sulfur compounds tested were captopril, sulindac, biotin, thioctic acid, zileuton, trithiocyanuric acid sodium salt, bis(carboxymethyl)trithiocarbonate, 1-(4'-methylphenacyl)-2-aminotetrahydrobenzothiazole, 2-aminoethylthiosulfonic acid, 6-n-propyl-2-thiouracil, 5-n-propyl-2-thiouracil, 5-carboxy-2-thiouracil, 4-hydroxy-2-mercapto-6-methylpyrimindine, 2-aminoethylisothiouronium bromide, and 2-ethyl-2-thiopseudourea hydrobromide. As these 15 sulfur compounds showed a wide range of activities and were not as effective as methionine for treatment of hearing loss caused by cisplatin administration, the data showed that different sulfur compounds have different effectiveness against hearing loss caused by cisplatin administration.

20. Unlike some other sulfur compounds and other amino acids, methionine can be reversibly oxidized and can act as a direct free radical scavenger. (Vogt 1995) This oxidative behavior may be a property that makes methionine an advantageous anti-ototoxicity agent. Kopke et al. did not disclose the oxidative properties of methionine, but only its role as a glutathione prodrug. In addition, methionine's properties of binding to the platinum center of cisplatin and glutathione substrate may add to its efficacy as a anti-ototoxicity agent for a variety of insults.

21. In addition, the data described in Attachment A show that D-methionine is highly effective as an otoprotective agent. In sum, D-methionine administration to experimental animals significantly decreased the threshold shift, significantly decreased the outer hair cell (OHC) and inner hair cell (IHC) loss and significantly

increased the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) in the cochlea as compared with the saline-treated control animals.

22. I hereby declare and state that all statements made herein are to my own knowledge true; and that all statements made on information and beliefs are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements will jeopardize the validity of the above-identified application or any patent issued thereon.

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Kathleen C.M. Campbell

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Jan. 12, 2005

Date

Attachment A

Twelve female adult *chinchilla laniger* were divided equally into two experimental groups. The two groups consisted of a saline control group and a pre-noise D-Met treatment group. Baseline hearing thresholds obtained through auditory brainstem response (ABR) were taken within two days of the initial noise exposure before and several hours after the last saline or D-Met injection before noise exposure.

Animals received D-Met (Sigma; 200mg/kg) or saline by intraperitoneal injection every twelve hours starting 48 hours prior to the noise exposure and again one-hour prior to noise exposure and then twice per day the following two days. Both groups had ABRs performed pre-noise, one-hour post noise, and once a week for three weeks. Shortly after the last ABR, animals were humanely euthanized, and the temporal bones were harvested and subsequently stained with a vital dye to indicate the presence of living hair cells.

ABR measurement

Animals were awake and lightly restrained in a plastic tube during the 30-minute recording procedure. Digitally generated stimuli consisted of tone pips (4 ms Blackman rise/fall ramp, 0 ms plateau, and alternating phase) at octave intervals 2, 4, 6, and 8 kHz. All acoustic stimuli were routed through a computer-controlled attenuator to an insert earphone (Etymotic Research ER-2). The sound delivery tube of the insert earphone was positioned approximately 5 mm from the tympanic membrane. Earphone sound delivery was calibrated using a coupler attached to the sound level meter approximating the distance from the earphone to the tympanic membrane. Six hundred samples were collected from the recording electrode, amplified (50,000-75,000 x), filtered (100-1500 Hz), and fed to an A/D converter computerized on a signal processing board. Stimuli at a rate of 23/sec were varied in 10-dB descending steps, until threshold was reached, then 5-dB ascending steps were presented to confirm threshold. Earphone inserts on the tested ear were removed, and control ABR runs during

which no sound was presented were determined for comparisons. Threshold was defined as the mid-point between the lowest level at which a clear response was evidenced and the next lower level where no response was observed.

Noise exposure

Our protocol was developed from the procedure of Hu et al. (1997). Specifically, an octave band noise centered at 4 kHz was generated by a standard audiometer (GSI 16), selected to white noise, routed through an attenuator (HP 350 D), a band-pass filter (Krohn-Hite 3550R), and a power amplifier (Crown D150A model 716) to an audiometric loudspeaker suspended directly above the animal's cage. The sound spectrum output of the system was confirmed using a Larson and Davis model 800B sound level meter, centering the octave bandwidth at 4kHz. In order to ensure consistent noise exposure conditions, the noise output of the system was monitored prior to each noise exposure using a sound level meter. Also, a pre-amplifier (Larson and Davis model 825), and a condenser microphone (Larson and Davis, LDL 2559), were positioned within the cage at the level of the animal's head for continuous monitoring during the exposure. Each animal was exposed continuously to the noise at a level of 105 dB SPL for six hours. During the noise exposure, the animal was unrestrained in a small wire cage with ad-lib water access. When the animals were not being exposed to noise, they were housed in a quiet animal colony.

Histologic examination

Following final auditory tests (i.e. at three weeks post noise exposure), the animals were heavily anesthetized with ketamine (30 mg/kg) and xylazine (1mg/kg) and decapitated. Each temporal bone was quickly removed from the skull. The cochlea was exposed and slowly perfused through the oval window and round window with a solution of 0.2 M sodium succinate and 0.1% nitrotetrazolium blue in 0.2 M phosphate buffer (pH=7.4 at 37° C). Samples were then immersed in the same solution for one hour at 37°C. Lastly, the cochlea

was rinsed with buffer and fixed with 4% paraformaldehyde for 24 hours. Cochleae were dissected and sections of the organ of Corti were mounted on glass slides and examined for hair cell loss under a light microscope (Olympus BH-2) at 400X magnification. Missing or non-viable hair cells were noted by the absence of blue vital stain in the area of inner and outer hair cells. An experienced but experiment-blinded observer counted missing hair cells over the length of the basilar membrane per cochlear turn utilizing specialized software. A cytocochleogram was developed for inner and outer hair cells for each cochlea and cytocochleogram means were computed and graphed.

Statistical analyses

A repeated measures ANOVA was used for analyzing the effect on hearing thresholds over time for each ear being treated with time and frequency as repeated measures. Post hoc tests were performed by the Newman – Keuls method with significance set at 0.05. An ANOVA was used to analyze the mean inner and outer hair cell counts in the 2, 4, 6, and 8 kHz regions of the cochlea and the means were compared for the two test groups. The Newman Keuls multiple comparison test (post hoc) was used to analyze the hair cell count data when significant differences were identified (0.05).

Audiometry Results

Baseline audibility thresholds ranged from a maximum of 26 to a minimum of 0 dB SPL at 2 to 8 kHz, respectively. These thresholds are consistent with independently published normative data (Hu et al., 1997; Kopke et al., 2000). Injection of D- Met prior to noise exposure did not significantly alter the baseline hearing thresholds ($p > 0.05$) (data not shown).

Auditory threshold shifts (group means for left and right ears) are displayed (Figure 1) as post-noise thresholds (dB SPL) ***minus*** baseline threshold (dB SPL). Means were plotted as a function of treatment group (saline-noise and D-Met antioxidant pre-treatment), over time (zero, one, two, three week) and by

threshold test frequency. The time zero hearing determination was one hour after cessation of noise exposure.

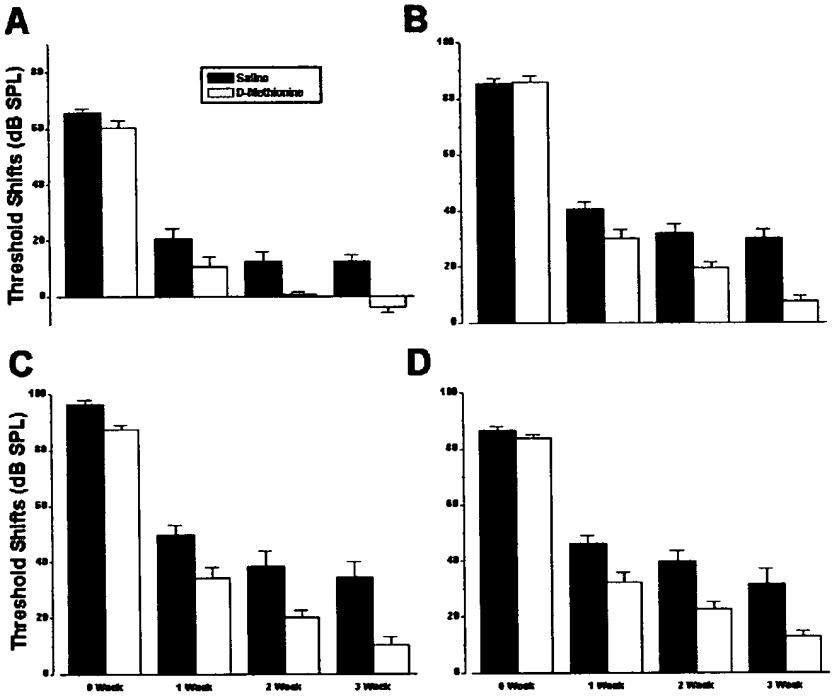

Figure 1

As shown (Figure 1A-D), six-hour sound exposure produced an initial threshold shift ranging from approximately 60-65 dB at 2 kHz to approximately 85-95 dB at the higher frequencies. There was evidence of recovery of threshold shift among both groups. However, the threshold shift recovery for the saline-noise group tended to stabilize over weeks two and three, resulting in no significant threshold improvement for any of the frequencies tested. In contrast, the D-Met treatment group showed a general trend toward improvement over the entire time period for all frequencies. There was an overall treatment effect for D-Met pre-treatment compared with the saline-noise condition ($p < 0.001$) for all tested frequencies beginning at week one.

As seen in Figure 1A-D, the permanent threshold shift (at three weeks) observed for the D-Met group was reduced compared to that of the saline-noise group. The threshold shift at three weeks for saline- noise exposed controls ranged between about 10 and 35 dB SPL from 2 to 8 kHz. The permanent threshold shift for the pre-treatment animals was significantly reduced to approximately 0-10 dB.

Hair cell counts

Figures 2A and B illustrate mean inner and outer hair cell counts, respectively, for saline and D-Met treated animals in a cytocochleogram which portrays missing hair cell percentages on the Y axis as a function of the measured percent distance from the cochlear apex. The associated frequency region of the cochlea is also plotted on the upper X-axis. When mean hair cell count data are considered for all animals, Figure 2A demonstrates that there is very little inner hair cell loss in D-Met treated animals compared to noise-saline controls. Figure 2B shows that the six hrs 4 kHz octave band noise exposure caused substantial outer hair cell loss in the saline-noise exposed animals, with maximal loss occurring between the 4 and 10 kHz region of the basilar membrane. Sixty to 70% of the outer hair cells were lost in the region between 3 and 10 kHz of the cochleae. Fewer inner hair cells were lost (maximum of 35%) in approximately the same region of the cochleae.

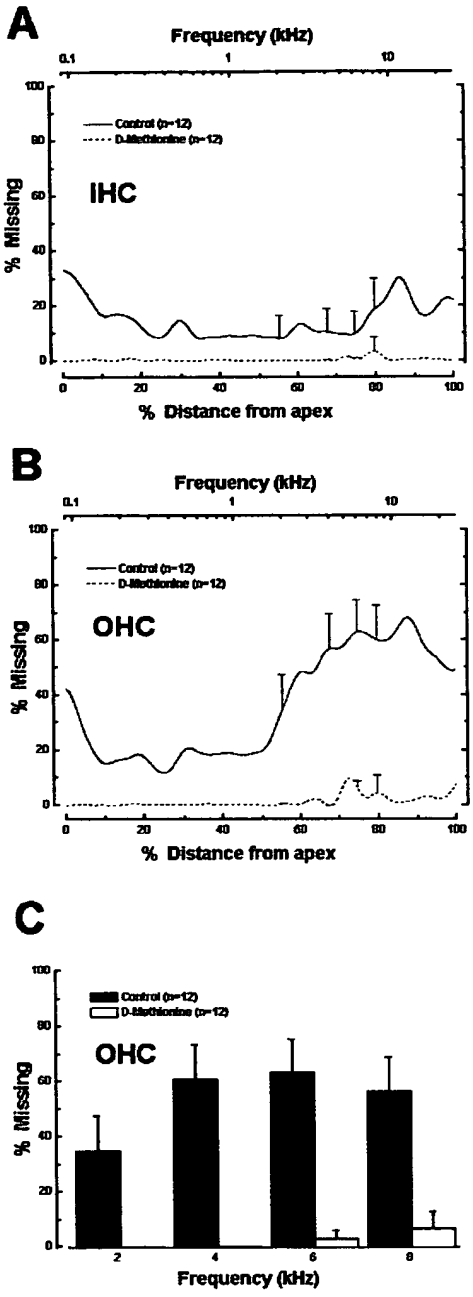
A
Frequency (kHz)
0.1
1
10
Control (n=12)
D-Methionine (n=12)
IHC
% Missing
% Distance from apex
B
Frequency (kHz)
OHC
% Missing
% Distance from apex
C
Control (n=12)
D-Methionine (n=12)
OHC
% Missing
Frequency (kHz)

Figure 2

In contrast, as shown in 2A and 2B, there was a substantial reduction in both inner and outer hair cell loss in the D-Met pre-treatment group compared to saline-noise (0 to 10 % outer hair cell loss verses 20 to 70%, 0 to 5% inner hair cell loss versus 10 to 35 %, respectively). The difference in mean outer hair cell loss at 2, 4, 6, and 8 kHz reached statistical significance for pre-treatment D-Met verses saline saline-noise groups ($p < 0.01$). The results for inner hair cell differences were not statistically different ($p>0.05$).

Oxidized (GSSG) and Reduced (GSH) Glutathione Levels

Data from studies conducted using the same chinchilla animal model, same D-met administration protocol and noise exposure stimulus (4 kHz octave band) as described above increases cochlear GSH levels, decreases cochlear GSSG levels and thus improves the cochlear GSH/GSSG ratio as a measure of oxidative stress. For Figure 3 (GSH/GSSG ratio- below) chinchillas were given D-met or saline according to the same protocol in these proposed studies and sacrificed after either .5, 2, 4, 6, or 8 hours of noise exposure. A single asterisk indicates significance difference between control and experimental groups at the .05 level. First results were analyzed with a between groups with repeated measures ANOVA for overall effects. We found a significant Groups x Exposure time interaction. Subsequent tests were corrected for overall type I error rate by using the Bonferroni correction and are depicted below.

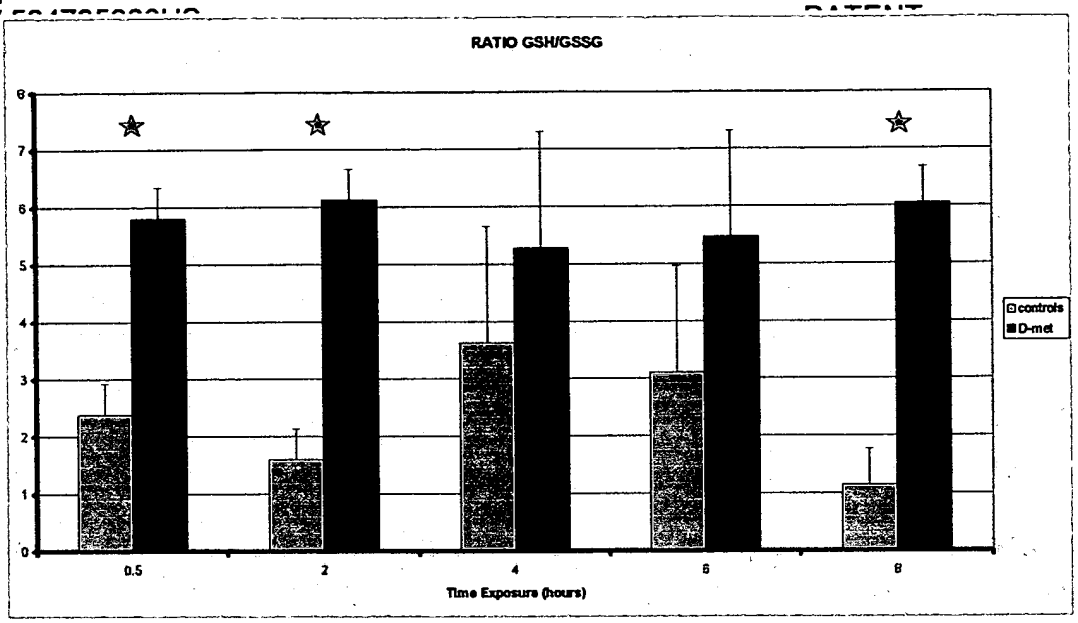

Figure 3

Rescue from noise exposure

We have also been conducting some studies in our lab investigating D-met rescue from the same noise exposure and chinchilla animal model as in the experiments described above. For these studies, D-met (200 mg/kg per dose) was administered starting 1 hour post noise exposure for either one dose, one dose plus two additional doses delivered BID (3 doses) or one dose plus 4 additional doses BID (5 doses). Because rescue dosing is not quite as effective as pre-administration we did not quite reach significance with 5 animals per cell but are conducting more experiments at this time. However the trend in the data was consistent for all ABR stimulus frequencies. Figures 4A and B ABR threshold results for 21 days post noise exposure using either saline injection (control) or 1, 3 or 5 post noise D-met injections in response to ABR stimulus frequencies of either 6 kHz (4A) or 8 kHz (4B). Similar findings were obtained for 2 and 4 kHz stimuli.

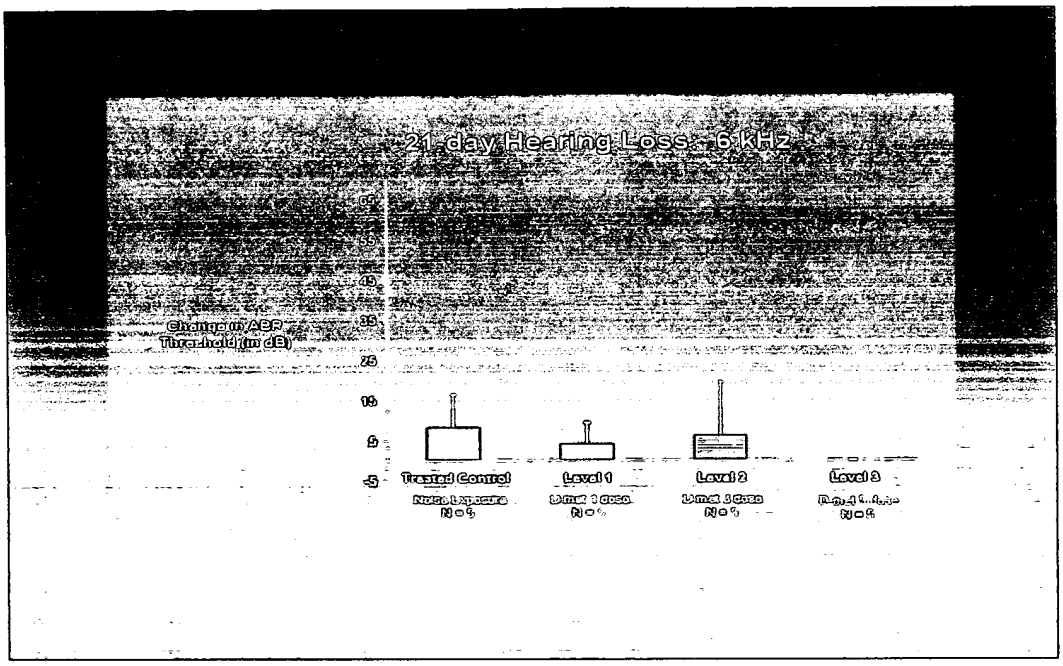

Figure 4A

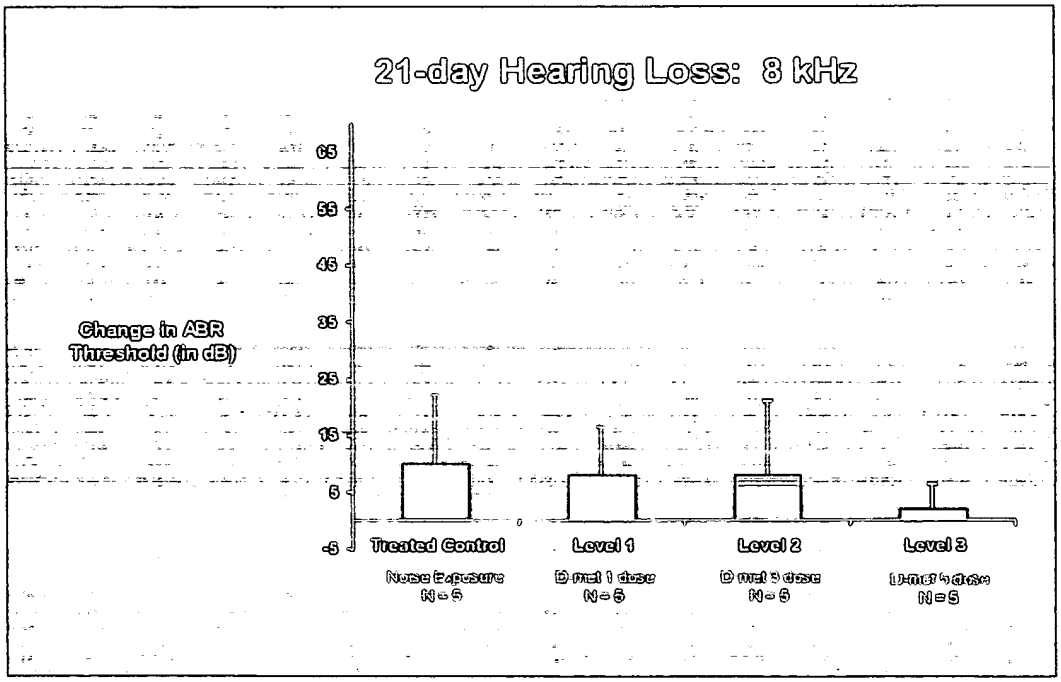

Figure 4B

References

Hu, B.H., Xiang, Y., McFadden, S., Kopke, R., Henderson, D. (1997) R-PIA attenuates noise-induced hearing loss in the chinchilla. Hear. Res. 113:198-206.

Kopke, R., Weisskopf, P.A., Boone, J.L., Jackson, R.L., Wester, D.C., Hoffer, M.E., Lambert, D.C., Charon, C.C., Ding, D-L, McBride, D. (2000) Reduction of noise-induced hearing loss using L-NAC and salicylate in the chinchilla Hear. Res.149: 138-146.

FEE TRANSMITTAL

Application Number 09/911,195 Art Unit 1614
Filing Date July 23, 2001 Confirmation No. 2942
Inventor(s) Kathleen C.M. Campbell
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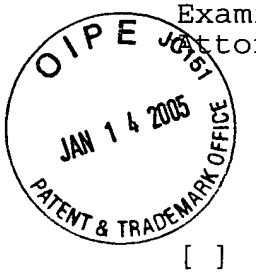

[X] Applicant claims small entity status.

METHOD OF PAYMENT

[ ] The Commissioner is hereby authorized to charge the indicated fees to Deposit Account No. 19-1345. The Commissioner is hereby authorized to charge any under payment or credit any over payment to Deposit Account No. 19-1345.

[X] Check Enclosed. The Commissioner is hereby authorized to charge any under payment or credit any over payment to Deposit Account No. 19-1345.

FEE CALCULATION

1. [ ] BASIC FILING, SEARCH AND EXAMINATION FEES
(Type: _____) Subtotal (1) $_____

2. [ ] EXCESS CLAIM FEES

Total Claims ___ - ___ (HP) = ___ x Fee ___ = $_____
Indep Claims ___ - ___ (HP) = ___ x Fee ___ = $_____
Multiple Dependent Claims Fee $_____
(HP = highest number of claims paid for)

Subtotal (2) $_____

3. [ ] APPLICATION SIZE FEE

Total Pages ____ - 100 = ____ ÷ 50 = ____ x $250 = $_____
(Application + Drawings) (round up to whole #)

Subtotal (3) $_____

4. [X] OTHER FEE(S)

[X] two month extension of time
[ ] Information disclosure statement
[ ] 37 CFR 1.17(q) processing fee
[ ] Non-English specification
[ ] Notice of Appeal
[ ] Filing a brief in support of appeal
[ ] Request for oral hearing
[ ] Other: _____

Subtotal (4) $ 225.00

TOTAL AMOUNT OF PAYMENT $225.00

John K. Roedel, Jr., Reg. No. 25,914 January 14, 2005
Telephone: 314-231-5400 Date

JKR/JSH/dep
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